AWARD NUMBER: W81XWH-12-1-0535

TITLE: A Multiplex Cancer/Testis Antigen-Based Biomarker Panel to Predict Aggressive Phenotype of Prostate Cancer.

PRINCIPAL INVESTIGATOR: Dr. Robert W. Veltri

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21205

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	30Sep2014 - 29Sep2015

4. TITLE AND SUBTITLE:

A Multiplex Cancer/Testis Antigen-Based Biomarker Panel to Predict Aggressive Phenotype of Prostate Cancer.

5a. CONTRACT NUMBER
JHFCONTRACT19

5b. GRANT NUMBER
W81XWH-12-1-0535

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
ROBERT VELTRI

E-Mail: rveltri1@jhmi.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
JOHNS HOPKINS UNIVERSITY
DEPARTMENT OF UROLOGY – THE
BRADY UROLOGICAL INSTITUTE
BALTIMORE, MD 21287

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The Cancer/Testis Antigens (CTAs) are a group of proteins normally confined to germ cells but aberrantly expressed in several cancers. The central hypothesis of this grant application is that a CTA-based biomarker can be used to discern LPCa from MPCa. In the first year of this grant, we determined gene expression of 22 candidate CTAs by Nanostring and validated by qRT-PCR. During the second year of the grant, we used ROC curve analysis and identified 8 CTA genes (CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2 and TTK), which expression pattern is significant different between aggressive and indolent tumors. For the third year of the grant, we evaluated the gene expression of these 8 CTAs in PCa and benign adjacent paired tissues from 24 patients. The only CTAs differentially expressed between non-cancer and cancer areas were PAGE4, SPAG4 and SSX2. For all the selected biomarker candidates, we obtained commercial antibodies from two sources and performed optimization using training TMAs containing normal and tumor prostate tissue. Quantification of the CTAs protein expression is being performed using an automated image system. We also performed CTA expression analysis in PCa cell lines (DU145, LNCAP, PC3, PC3 Epi, PC3 EMT and BPH1) by qRT-PCR and Western Blot. Absence of gene expression correlated with no protein translation.

15. SUBJECT TERMS
Prostate cancer; metastatic prostate cancer; cancer/testis antigens (CTA); biomarker; gene expression; nCounter; Nanostring; qRT-PCR

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	14	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths among men in the US (Siegel et al., 2014). The introduction of prostate specific antigen (PSA) test has greatly aided to the early detection of PCa. Detectable levels of PSA are the earliest sign of recurrent disease after radical prostatectomy (RP) (Pound et al., 1999). Besides its sensitiveness, it is estimated that 23-44% of patients submitted to RP will progress with detectable PSA levels and will never present recurrence (Draisma et al., 2009). Thus, a clinical dilemma today in the management of PCa is to distinguish men with aggressive disease who need definitive treatment from men with indolent disease not requiring immediate intervention. As PSA screening is not capable of discriminating between low risk and aggressive PCa the identification of novel biomarkers is critical to offer patients adequate treatment following RP. The central hypothesis of this study is that a CTA-based biomarker can be used to discern PCa patients with aggressive disease and hence would need definitive treatment from those in whom it is less likely to recur and would not require immediate intervention. The Cancer/Testis Antigens (CTAs) are a unique group of heterogeneous proteins that are normally confined to germ cells in normal testis and placenta, but aberrantly expressed in several types of cancers (Scanlan et al, 2004). Unfortunately, their potential as biomarkers in PCa has not been rigorously explored and a coordinated expression pattern of the CTAs associated with tumor grade/stage has not been demonstrated to date for any type of cancer. This hypothesis will be addressed with the following specific aims: 1) to identify candidate CTAs that are differentially expressed in clinically organ-confined PCa and metastatic PCa tissues; 2) to develop the expression profile of CTAs to predict the aggressiveness of PCa using the CTA-based nCounter Gene Expression Assay; and 3) To test whether the CTAs expression profiling can differentiate the ‘aggressive’ versus ‘indolent’ PCa using blinded samples. In addition to the blinded sample sets, this specific aim will also explore the possibility of using peripheral blood cells that contain circulating tumor cells for assaying the CTAs expression profiling.

2. KEYWORDS

1. Prostate cancer
2. Metastatic prostate cancer
3. Cancer/testis antigen
4. CTA
5. Aggressive
6. Biomarker
7. Gene expression
8. nCounter
9. Nanostring
10. qRT-PCR

3. OVERALL PROJECT SUMMARY

Summary of Tasks in SOW

Tasks	Summarized aims	Time
Major Task 1 Subtasks 1 and 2 Year 1	CTA gene expression analysis by nCounter (Nanostring) and validation by qRT-PCR.	Year 1
Major Task 2 Subtasks 1 and 2 Year 2	1. Confirm CTA expression pattern in 24 tumor extracts of PCa (paired non-tumor and tumor cases). 2. Identify and optimize commercial sources of specific antibodies to perform quantitative IHC using samples organized in training TMAs available at Dr. Veltri's laboratory.	Year 2
Major Task 3 Subtasks 1, 2 and 3	1. Evaluate CTA expression using qIHC for the candidates selected in a TMA containing PCa cases organized according to Gleason score. 2. Evaluate CTA expression using qIHC for the candidates selected in a TMA containing PCa cases organized according to biochemical recurrence. 3. Use uni- and multi-variate logistic regression analysis to create a panel of aberrant expressed CTA that are capable of discriminate and/or predict recurrence for PCa.	Year 3

Summary of year 1

In the first year of the project (Dr Prakashi Kulkarni was the PI), CTA expression was evaluated using nCounter (Nanostring). A group of 20 localized PCa samples and 20 metastatic cases obtained from Dr. Robert Vessella at University of Washington (Seattle, WA) were used for CTA gene expression analysis. The nCounter results were validated by qRT-PCR, attesting that this multiplex approach may be appropriate for the identification of CTA genes differentially expressed in localized versus metastatic disease.

After completion of the gene expression analysis a change in the project design was requested. In October 2013, as the suggestion of Dr. Kulkarni, it was requested that the PI in charge of the grant be changed from Dr. Prakash Kulkarni to Dr. Robert W Veltri. This change was accepted by the CDMRP in **April, 2014 and W81XWH-12-1-0535 award** was revised. During this process the project was put on hold until a final decision could be made. As soon as the change was accepted, a new postdoctoral fellow (Dr. Luciane T. Kagohara) was hired (July, 2014) and the CTA

W81XWH-12-1-0535 project restarted. Due to the delay caused by changes in the project, the Year 2 tasks were delayed and were performed during Year 3.

Summary of year 2 (July/2014 to October/2014)

To identify the best CTA candidates as biomarkers for aggressive prostate cancer (PCa) we performed statistical analysis of the data obtained for CTA gene expression by Nanostring and qRT-PCR of the 20 localized prostate cancer (LPCa) and 20 metastatic prostate cancer (MPCa) specimens. PRISM software was used to calculate Receiver operator characteristic (ROC) curves to identify a cutoff ratio above the highest control ratio observed for each gene to set specificity at the percentage that maximizes the number of samples correctly classified. Using these cutoff ratios determined by the statistical analysis we then compared the means and verified which CTA pattern of expression were able to discriminate LPCa and MPCa. Combining Nanostring and qRT-PCR ROC curve analysis, our best candidates are: *CEP55, NUF2, PBK, RQCD1, SPAG4, SSX2, TTK* and *PAGE4*.

Year 3 (November 2014 to October 2015)

CTA gene expression analysis in benign adjacent and PCa paired samples

Twenty-four paired cases of adjacent benign and tumor tissue (total of 48 samples) collected from PCa patients submitted to radical prostatectomy were available at Dr. Veltri's laboratory. RNA was already obtained from the tissue samples, quantification and quality verification was also performed.

One microgram of total RNA was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad). The PCR reactions were performed with 0.2 µl of cDNA template in 25 µl of reaction mixture containing 12.5µl of iQ SYBR Green Supermix (Bio-Rad) and 0.25 µmol/L each primer. PCR reactions were subjected to hot start at 95ºC for 3 minutes followed by 45 cycles of denaturation at 95ºC for 10 seconds, annealing at 60ºC for 30 seconds, and extension at 72ºC for 30 seconds using the CFX96 Real-Time PCR Detection System (Bio-Rad). Analysis and fold differences were determined using the comparative threshold cycle method. ACTB was the housekeeping gene used for normalization.

PRISM software was used to calculate Receiver operator characteristic (ROC) curves to identify a cutoff ratio above the highest control ratio observed for each gene to set specificity at the percentage that maximizes the number of samples correctly classified. Using these cutoff ratios determined by the statistical analysis, we then compared the means and verified which CTA pattern of expression were able to discriminate normal (benign adjacent) from cancer cases.

All ROC curve analysis is summarized in Table 1 and Figure 1 shows the scatter plots for the CTA genes evaluated.

There was no significant difference in the expression pattern of *CEP55, NUF2, PBK, RQCD1* and *TTK* when comparing non-tumor and tumor samples. *PAGE4* was down-regulated in the benign adjacent samples when compared to PCa areas. We previously described reduced levels of *PAGE4* in LPCa vs. MPCa. The CTAs *SPAG4* and *SSX2* were initially found down-regulated in LPCa when compared to MPCa. However, in our cohort of paired benign and tumor samples these CTAs were down-regulated in the tumor areas.

The observed pattern of CTA expression between benign adjacent and tumor areas from the same patient might be an indication that even in the absence of phenotypic changes other effects as a consequence of the presence of cancer cells (e.g. inflammatory reaction in response to the cytokines released by cancer cells) result in aberrant gene expression.

Quantitative Immunohistochemistry: antibody selection and reaction optimization

IHC reactions for the 8 selected CTAs was performed following a protocol well established by Dr. Veltri's research group. Briefly, deparaffinization of tissue sections was performed in xylene and followed by re-hydration in serial washes in ethanol (100%, 75%, 50% and 25%). Antigen retrieval was performed under heat and adequate pH. After that, steps for endogenous peroxide activity and unspecific protein blocking were performed at room temperature. Incubation with primary antibody was performed overnight at 4ºC using pre-optimized dilution. Secondary antibody in a 1:200 dilution was incubated for one hour at room temperature. Staining was performed using DAB substrate solution and counter staining in hematoxylin. Quantitative IHC (qIHC) utilizes MediaCybernetics Inc. ImagePro 9.1 to capture immunohistochemically stained tissue slides images that are scanned with Aperio Scanning microscope at 20X and the TIFF image files of tumor area and normal glandular area are extracted using Aperio Image Scope Software in a non-compression format. Then the ImagePro 9.1 software applies primarily pixel intensity and total area IHC features to quantify the results and the outcome is a continuous variable for statistical analysis.

IHC reactions for *CEP55, NUF2, PBK, RQCD1, SPAG4, SSX2, TTK* and *PAGE4* were performed using primary antibodies from 2 sources: Sigma-Aldrich and Abcam. All antibodies went through an optimization step to determine the proper ideal dilution for the reactions. All optimization reactions were performed in a training TMA containing 8 cores of PCa cases and a series of 16 normal tissues from different origins. Once the ideal dilution for each primary antibody was reached the final IHC reactions were performed on TMAs 681 and 682. Table 2 shows the concentration for each primary antibody selected from the different commercial sources.

Figure 2 shows representative pictures from the TMA slides scanned on Aperio Image Scope for the reactions performed with Sigma-Aldrich antibodies. For some of the antibodies, staining of stromal tissue was observed. To verify if it was unspecific antibody binding we performed IHC reactions with a different source of antibodies (Abcam). Reactions were already performed and image scanning is underway. We will perform IHC staining quantification even in the presence of stromal staining in an attempt to verify if any association between clinical and pathological features and presence of stromal CTA expression will be observed. Since CTAs are highly expressed during embryogenesis differentiation, presence of tumor stromal expression of these biomarkers might be a suggestion that in some cases the stromal cells are still under differentiation and it can have a correlation with PCa stage.

CTA gene and protein expression in PCa cell lines

In parallel to CTA expression in prostate tumor and normal tissues, we evaluated the expression profile of *CEP55, NUF2, PBK, RQCD1, SPAG4, SSX2, TTK* and *PAGE4* in PCa cell lines. mRNA and protein expression were determined by qRT-PCR and Western Blot, respectively.

We performed qRT-PCR following the protocol described above. Cell lines were cultured under proper conditions and RNA was extracted following the Tryzol reagent (Invitrogen) protocol, according to manufacturer's instructions. Total protein for Western Blot expression analysis was obtained using RIPA buffer (Thermo Scientific), following manufacturer's instructions. SDS-

PAGE electrophoresis used 12% Bio-Rad pre-cast gels, proteins were then transferred to PVDF membranes using the Turbo Gel Transfer. After blocking with 5% milk, primary antibody was incubated overnight, followed by respective secondary fluorescent antibody incubation. Membranes were scanned using the Li-Cor system.
For this analysis we selected 6 cell lines: BPH1, DU145, LNCAP, PC3, PC3 Epi and PC3 EMT. Gene expression levels (bar graphs) and protein expression (SDS-PAGE) for all CTAs and cell lines selected are represented on Figure 3. Protein expression reflected mRNA levels in the cell lines: positive gene expression resulted in protein detectable levels, while no or low levels of mRNA reflected in negative protein expression. The only exception was for *PBK*. Even in the presence of mRNA expression, no protein was observed in any of the cell lines. We are still investigating if the cell lines have no protein expression or if the antibody against PBK is not binding to the antigen.

Next experiments

In the previous annual report one of our next steps was to obtain and perform qIHC in another TMA (PSA Progression) that comprises normal and prostate tumor samples from patients with and without biochemical recurrence and is composed of 726 cases in total. However, consulting the clinical and pathological information from the cases included in the mentioned TMA blocks, we found that there were not information regarding to development of metastasis by the patients included in the TMA. As our project main aim is to identify biomarkers for aggressive PCa, our endpoint is occurrence of metastatic disease. We were informed by the PCBN (Prostate Cancer Biorepository Network) that a new TMA for which the endpoint is metastatic PCa is under development and we decided to wait the construction of the blocks to be done to submit a request to obtain new TMAs for validation of our biomarkers.
In an attempt to determine if CTA expression by the PCa cells can induce cellular mediated immune response, we will evaluate the presence of lymphocytic infiltration in the tissue samples included in the TMAs. Presence of immune cellular response will be determined by the detection of lymphocytic infiltrate by IHC against markers for T cells (CD3+, CD8+ and FOXP3+). CD3 is a pan T cell surface antigen that will be used to identify non-specific lymphocytic infiltration. CD8+ cells are critical for tumor-specific cellular immunity. CD4+ FOXP3+ cells are T regulatory cells with immunosuppressive properties. Identifying these populations of T cells in the tumor will be an indication of an immune response against the PCa cells. The presence of lymphocytic infiltration will be correlated with CTA expression. To further evaluate how CTAs might be interfering with PCa patients' immune response we will evaluate the expression of blockage checkpoint components (PD-1, PD-L1 and CTL4), with that we expect to verify if the expression of these molecules are associated with CTA expression which might result in studies for the development of new targeted cancer immunotherapy or new combinations of immunostimulatory antibodies and CTA-based cancer vaccines.

4. KEY RESEARCH ACCOMPLISHMENTS

- CTA gene expression analysis in paired benign adjacent and tumor tissue from PCa patients did not show significant differences for *CEP55, NUF2*, *PBK*, *RQCD1* and *TTK*. For PAGE4, SPAG4 and SSX2 up-regulation was detected in samples obtained from non-tumor areas of the prostate.

- IHC optimization for the CTAs *CEP55, NUF2, PBK, RQCD1, SPAG4, SSX2, TTK* and *PAGE4* was performed and accomplished using two different sources of primary antibodies. Some of the IHC reactions showed tumor stromal staining, even in the absence of tumor expression of the CTA, this feature will be considered during the IHC quantification to verify if there is any correlation with clinical and pathological characteristics of the patients.

- mRNA and protein CTA expression analysis in PCa cell lines were concordant. Absence of detectable gene expression by q-RT-PCR reflected in absence of protein expression (evaluated by Western Blot). No association between CTA expression and cell line metastatic potential was observed (i.e. no association between CTA expression and aggressiveness was observed).

5. REPORTABLE OUTCOMES

Nothing new to report.

6. CONCLUSION

The results we found up to this point of the study are clinically and biologically relevant, since little is known about the role of CTAs in PCa. The pattern of expression of the group of genes we tested were shown to be different between two extreme phenotypes, very aggressive (metastatic tumors) and indolent tumors (low grade small tumors, low stage). However, our current expression analysis in tumor and the paired benign adjacent prostate tissue did not show significant differences in the levels detected. It suggests that the aberrant CTA gene expression might be a phenomena observed in the whole prostate tissue even in the absence of malignant phenotype, probably as result of an inflammatory reaction in response to chemokines secreted by some of the malignant cells.

CTA protein quantification analysis by IHC is underway. Nevertheless, we already noticed that in a significant number of cases there is presence of stromal cell staining. We will quantify the staining in these areas and evaluate if there is association with disease prognostic factors. CTAs are normally expressed during embryogenesis and in PCa tissue it might be a suggestion that the stromal cells are still going through differentiation and we want to verify if there is any correlation with the tumor stage.

We observed that the CTA expression profile changes among the different PCa cell lines. It was not observed any association between the metastatic potential of the cell lines and the pattern of expression of the CTAs selected.

7. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

- Multi-Institutional Prostate Cancer Program Retreat in Ft. Lauderdale, FL - March 15-17, 2015.
  "Cancer/testis Antigen Biomarker Expression Pattern Can Discriminate Localized vs. Metastatic Prostate Cancer."

- 2015 American Association for Cancer Research (AACR) Annual Meeting in Philadelphia, PA – April 16-20, 2015.
  "Cancer/testis Antigen Expression Pattern is a Potential Biomarker for Prostate Cancer Aggressiveness."

- Manuscript is being prepared for submission.

8. INVENTIONS, PATENTS AND LICENSES

Nothing to report.

9. OTHER ACHIEVEMENTS

Nothing to report.

10. REFERENCES

Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014 Jan-Feb;64(1):9-29.

Pound CR, Partin AW, Eisenberger MA, Chan DW, Pearson JD, Walsh PC. Natural history of progression after PSA elevation following radical prostatectomy. JAMA. 1999 May 5;281(17):1591-7.

Draisma G, Etzioni R, Tsodikov A, Mariotto A, Wever E, Gulati R, Feuer E, de Koning H. Lead time and overdiagnosis in prostate-specific antigen screening: importance of methods and context. J Natl Cancer Inst. 2009 Mar 18;101(6):374-83.

Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. Cancer Immun. 2004 Jan 23;4:1.

11. APPENDICES

Nothing to report.

12. SUPPORTING DATA

Table 1 – Summary of ROC curve analysis to evaluate the utility of CTA levels to distinguish non-tumor from tumor tissue obtained from the same patient.

	CEP55	***NUF2***	***PAGE4***	***PBK***	***RQCD1***	***SPAG4***	***SSX2***	***TTK***
Area under curve (AUC)	0.58	0.69	0.98	0.58	0.59	0.83	0.74	0.55
Sensitivity	13.04%	13.04%	95.65%	0.00%	52.17%	73.91%	78.26%	100.00%
Specificity	92.00%	100.00%	92.00%	100.00%	64.00%	76.00%	64.00%	0.00%
Positive predictive value	60.00%	100.00%	91.67%	na	57.14%	73.91%	66.67%	53.66%
Negative predictive value	53.49%	55.56%	95.83%	53.19%	59.26%	76.00%	76.19%	na
Correctly classified	54.17%	58.33%	93.75%	53.19%	58.33%	75.00%	70.83%	53.66%

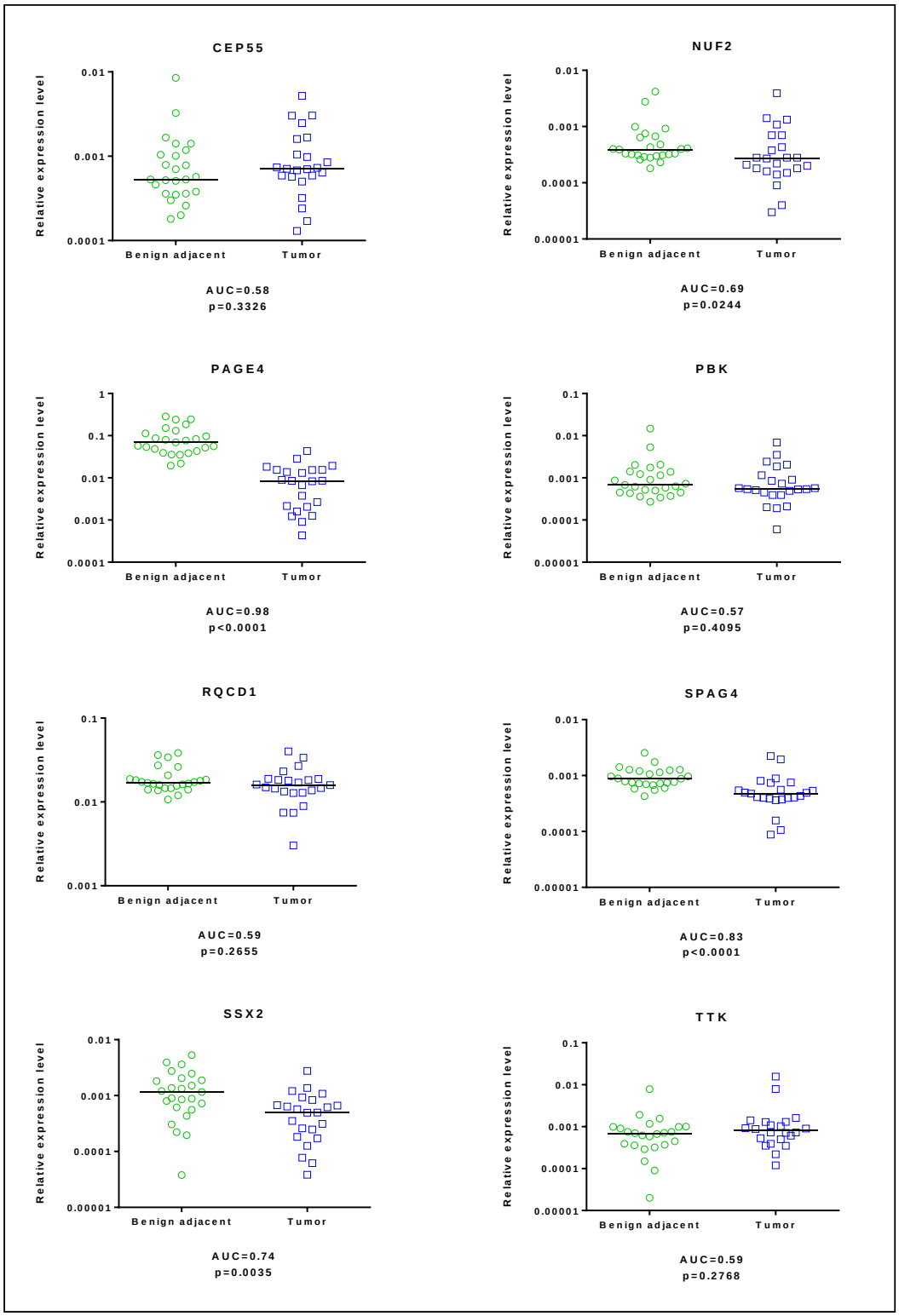

Figure 1 – CTA gene expression analysis. Scatter plots representing the relative gene expression levels for each CTA. Green circles correspond to benign adjacent tissue and blue squares to tumor areas. AUC was obtained by ROC curve analysis and p values from Mann-Whitney non-parametric test. Those CTAs with AUC>0.70 (PAGE4, SPAG4 and SSX2) are those which expression levels can be used to separate tumor areas from benign adjacent prostate tissue.

Table 2 – IHC primary antibody source and optimized dilution for TMAs 681 and 682.

CTA	Primary antibody dilution	
	Sigma-Aldrich	Abcam
CEP55	1:250	1:2000
NUF2	1:75	1:25
PAGE4	1:1000	1:50
PBK	1:200	1:10
RQCD1	1:10	na *
SPAG4	1:200	1:300
SSX2	na *	1:50
TTK	1:250	1:200

* SSX2 IHC was performed only using Abcam antibody. Sigma-aldrich showed very strong unspecific staining.
** RQCD1 IHC was performed only using Sigma-Aldrich antibody. Abcam showed very strong unspecific staining.

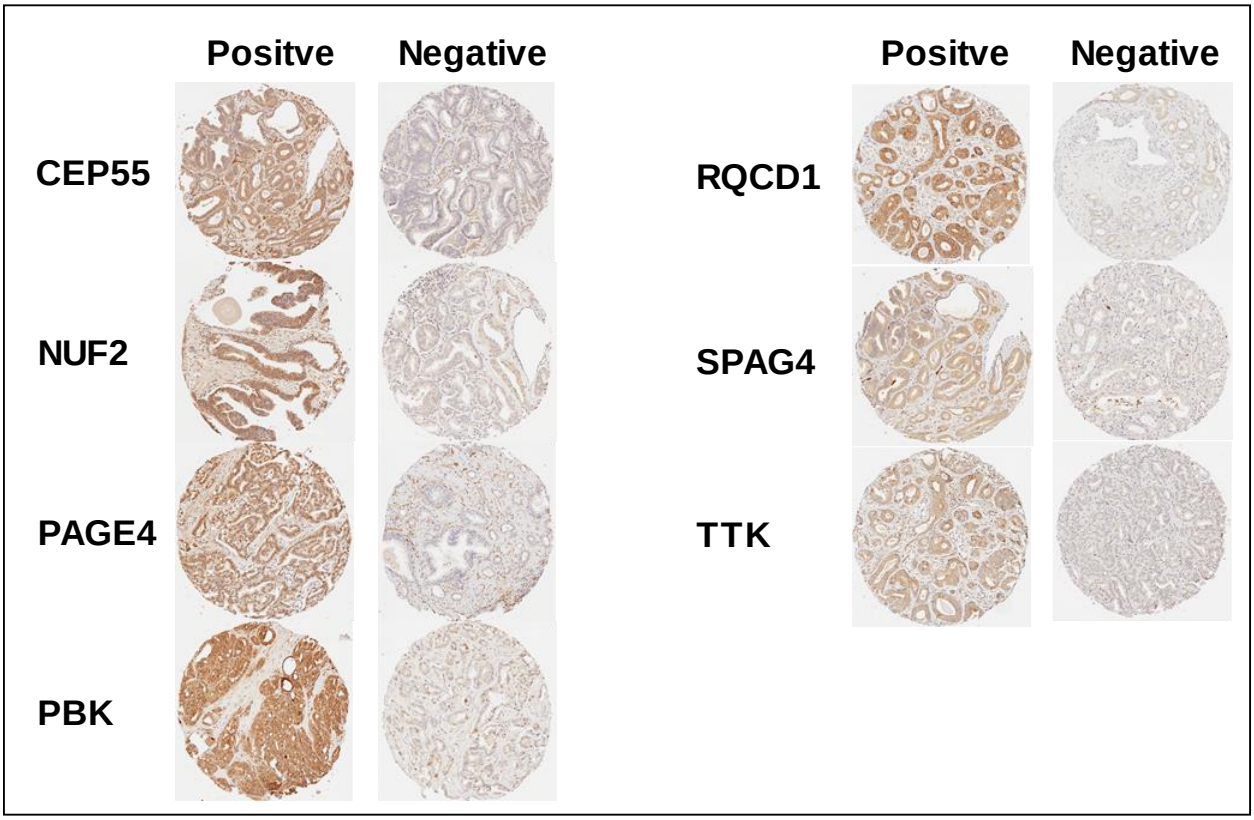

Figure 2 – IHC scanned images for the detection of CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4 and TTK performed using primary antibody produced by Sigma-Aldrich. SSX2 IHC reaction showed very strong unspecific staining even after attempts using high antibody dilution. For some of the biomarkers, stromal cell staining can be noticed even when the cancer cells show no expression or very low levels of the CTA. The same reactions were performed using Abcam primary antibodies and TMA slides are in the scanning process by the TMA and Pathology core facility at Johns Hopkins Medical Institution.

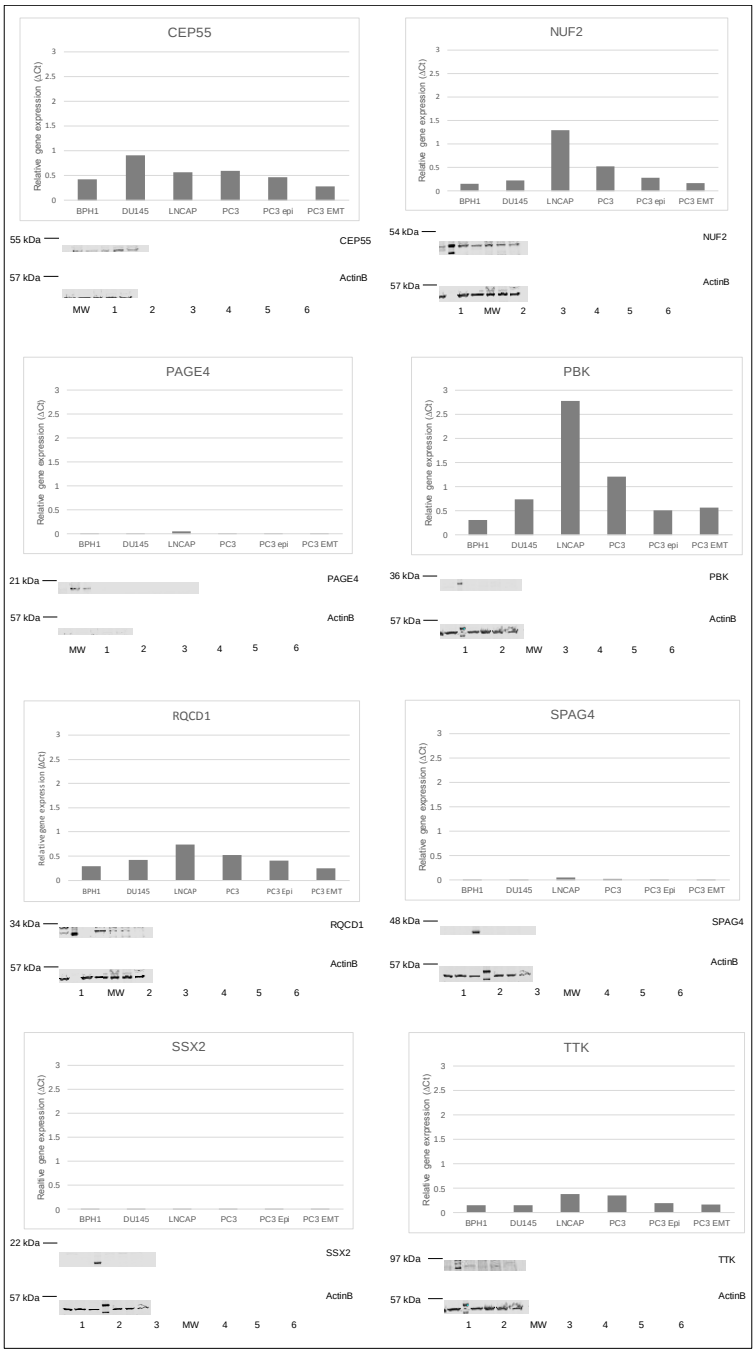

Figure 3 - Relative gene mRNA expression (qRT-PCR) and protein expression (Western blot) levels of CTAs in PCa cell lines. 1-BHP1; 2-DU145; 3-LNCAP; 4-PC3; 5-PC3 Epi; 6-PC3 EMT; MW-protein molecular weight marker. CTA mRNA and protein expression shows concordance: absence of mRNA expression results in no detectable levels of protein. The only exception is PBK that shows significant gene expression but no protein could be detected by Western Blot. We are further investigating the possible causes for no detectable protein expression (e.g. inefficient antibody binding to the epitope).